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Importance of the Bcl-2 family in cell death regulation

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Abstract. Bcl-2 was first identified as a novel transcript associated with the t(14;18) chromosomal breakpoint which occurs in most follicular lymphomas. The deregulated expression of bcl-2 was found to contribute to multistep neoplasia through the suppression of cell death, or apoptosis, in transgenic mouse models. Bcl-2 was subsequently shown to be normally expressed in a variety of tissues and to significantly inhibit the induction of apoptosis in many experimental systems. Bcl-2 is now known to be structurally similar to other proteins, in particular within the domains referred to as BH1 and BH2. This multigene family of cell death regulators includes members which enhance rates of apoptosis, including bcl-x_S and bax, and those which inhibit apoptosis, including MCL-1 and bcl-x_L. Members of the bcl-2 family physically interact with other proteins, including other family members and these interactions appear to modulate their function. The mechanism(s) by which bcl-2 family members regulate cell death remain in large part unknown, although recent evidence suggests that bcl-2 may interfere with cellular signalling events involved in apoptosis induction.
Key words. Bcl-2; bax; bcl-x; apoptosis; cell death.

Apoptosis, or programmed cell death (PCD), is an important process which is necessary for normal embryological development and the maintenance of homeostasis [1]. The deregulation of apoptotic cell death appears to contribute to the pathogenesis of certain diseases such as T-cell depletion associated with acquired immune deficiency syndrome [2], neural degeneration [3, 4], and cancer [5].

Much of our recent understanding concerning the molecular regulation of cell death originates with the bcl-2 gene family. Bcl-2 was first identified as a novel transcriptional element associated with the t(14;18) chromosomal translocation breakpoint in follicular lymphoma [6–10]. Cell death suppression by bcl-2 was first characterized in hematolymphoid cells [11–13] and subsequently in various other cell types. It is reported that bcl-2 can block cell death caused by chemotherapeutic drugs [11, 14], ultraviolet (UV) radiation, heat shock [15], some viruses [12, 16], free radicals [17–19], Ca^{2+} [20–22], tumour necrosis factor [23–26] and withdrawal of growth factors [27]. However, there are inducers of apoptosis which appear to be independent of bcl-2 function, like cytolytic T-cell killing [28], and withdrawal of specific lymphokines [27, 29]. Observations which suggest cell death may be induced by mechanisms which are referred to as 'bcl-2 independent' must be interpreted with caution, in that such inducers may function downstream of bcl-2 or, alternatively, result in upregulation of dominant acting death effector molecules, in an otherwise bcl-2-regulated pathway.

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Several proteins have recently been identified, on the basis of sequence similarities and physical interactions, which are similar to bcl-2. It is now established that bcl-2 is a member of a multigene family. This emerging protein family shares at least two highly conserved regions, which have been referred to as bcl-2 homology 1 and 2 (BH1 and BH2) domains [30, 31] (figure 1). Included among the human bcl-2 family members are bax, bcl-x, bak, bad and MCL-1. The bcl-2 family consists of proteins which function to inhibit apoptosis, including bcl-x_L [32], MCL-1 [33], and bcl-2, and those which function to accelerate the rate of apoptosis once it has been induced, including bax [30], bcl-x_s [32] and bad [34]. The identification of multiple members of the bcl-2 family, which demonstrate both nonoverlapping function as well as tissue distribution (table 1), emphasizes that cell death, similar to cell proliferation, is a complexly regulated process.

Bcl-2

The *bcl-2* gene was first identified as a novel transcriptional element associated with the t(14;18) chromosomal translocation which occurs at high frequency in follicular lymphoma [6–10]. The *bcl-2* gene maps to 18q21 and is expressed at inappropriately high levels in those lymphomas possessing the t(14;18). The human *bcl-2* gene consists of three exons and spans approximately 230 kb [35]. The *bcl-2* open reading frame spans exons 2 and 3 and encodes a 25-kD integral membrane protein.

The transcription of *bcl-2* is initiated by two promotors, P1 and P2 [35]. The P1 promoter, considered the most

Table 1. Tissue distribution of bcl-2 family proteins*.

		Bcl-2	Bax	Bcl-x	MCL-1
GI tract					
Salivary glands					
	mucinous cells	+	–	+	nd
	serous cells	nd	+	+	nd
Esophagus		+	+	+	nd
Stomach					
	gastric pit epithelium				
	mucous epithelium	+ +	+	+ +	+ +
	parietal, chief cells	+	+	+	+
Pancreas	exocrine cells	–	+ + +	+	+
	islet cells	+	–	–	nd
Small intestine					
	villi				
	paneth cells	–	+ + +	+ +	–
	absorptive epi. cells	+	+	+ +	+ + +
	crypt cells	+	nd	+	+
Colon	crypts				
	apical epithelial cells	+	+ +	+	+ + +
	basal epithelial cells	+ +	+	+	–
Liver	hepatocytes	+	+ +	+	–
Respiratory tract					
Lungs	pneumocytes of alveoli	–	– +	+	–
	bronchial epithelium	+	+	+ +	+
Cardiovascular system					
Heart	myocardium	+	+	+	+ +
Arteries	endothelium	–	+	–	–
	media smooth muscle	+ +	+ + +	+	+
	adventitia	–	–	–	–
Musculoskeletal					
Cartilage	chondrocytes	nd	–	–	nd
	osteoblasts	–	–	+	–
	skeletal muscle	+	+ +	+ +	+
Renal system					
Kidney	glomeruli	–	–	–	–
	Bowman's capsule	–	+ +	–	nd
	proximal tubules	+	+ +	+	–
	loop of Henle	+	+ +	+ +	+ +
Distal	tubule	–	+ +	+ +	+ +
Ureter	uroepithelium	+ + +	+	+	nd
Bladder	epithelium	+ + +	+	+	+ +
Skin					
	keratinocytes				
	basal cell layer	+ + +	+	+	+
	spinous layer	+ +	+	+ +	+
	granular layer	+ + +	+	+ +	+ + +
	cornified layer	– +	+	+	
Hematolymphoid					
	thymus				
	eithelial cells	+	+ + +	+	+ + +
	cortical thymocytes	–	– to +	+ +	– to + +
	medullary thymocytes	+ + +	– to +	+	+
Lymph nodes					
Lymphocytes in:					
	germinal center	–	+ + +	–	+ + +
	mantle zone	+ + +	–	–	+
	interfollicular	+	+ + +	+	+ + +
	histiocytes	–	+ +	–	nd
Spleen					
	white pulp	+ + +	+	–	+ +
	red pulp	+	–	–	+
Bone marrow					
	myeloid precursors	+ +	+ + +	+	+
	erythroid precursors	+ +	+ +	+ +	+
	megakaryocytes	+ +	+	+ +	+
Nervous system					
	peripheral	+ + +	+ + +	+ +	+
	central		– to + + +	– to + + +	+ +
Reproductive tissues					
Male					
	prostate basal	+ + +	+	+	+
	secretory	–	+ + +	+ +	+ +
	testis	+ + +	+	+	– to + + +
Female					
	myometrium	+	+	+	+ + +
	endometrium	+ + +	+	+	+
	ovarian	+	+	+	+
	mammary gland	+ + +	+ +	+ +	+

*Protein levels were assessed using immunohistochemical analysis of tissue sections for bcl-2 (50), bax (51), bcl-x [109] and MCL-1 [88, 110]. Levels of expression are normalized to appropriate internal standards of comparison. nd = not determined.

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                                           Domain A
                         10             20        30        40           50        60
                         *     *     *     *     *     *     *     *     *     *     *
bcl-2          MAHAGRTGYD N   REIVMKYIH YKLSQRGYEW DAGDV  GAAPP GAAPAPGIFS SQPGHTPHP
bclx long            MSQS N   RELVVDFLS YKLSQKGYSW SQFDV  EENRT EAPEETEAER ETPSAINGN
bclx short           MSQS N   RELVVDFLS YKLSQKGYSW SQFDV  EENRT EAPEETEAER ETPSAINGN
bax       MDGSGEQPRGGGPTS     SEQIMKTGA LLLQG-----------------------------------
bak                                MASGQGPGP PRQECGEPALP SASEEQVAQD TEEVFRSYVF
A1                                                                  MAESELMHI
mcl-1           MFGLKRNAVI GLNLYCGGAGL GAGSGG---------ESGNNTS TDGSLPST-- PPPAEEEEDE
bad            MGTPKQPSLAP AHALGLRKSDP GIRSLGSDAG GRRWRPAAQSM FQIPEFEPSE QEDASATDRG

                                                                       BH 3
                70        80         90        100           110              120
bcl-2          ASRDPVARTS PLQTPAAPGA AAGPALSPVP PVVHLA  LRQA GDDFS  RRYRG DFAEMSSQLH
bclx long      PSWHL-ADSP AVNGATGHSS SLAREVIPMA AVKQA-  LREA GDEFE  LRYRR AFSDLTSQLH
bclx short     PSWHL-ADSP AVNGATGHSS SLAREVIPMA AVKQA-  LREA GDEFE  LRYRR AFSDLTSQLH
bax            FIQDRAGRMG GE-APELA-L DPVPQDASTK KLSEC-  LKRI GDELD  SNM-E -LQRMIAAVD
bak            YRHQQEQEAE GVAAPADPEM VTLPL-QPSS TMGQVG  RAII GDDIN  RRYDS EFQTMLQHLQ
A1             HSLAEHYLQY VLQVPAFE-S APSQAC-RVL QRVAFS  VQKE VEKNL  KSYLD DFH-VESI-D
mcl-1          LYRQSLEIIS RYLREQATGA KDTKPMGRSG ATSRKE  LRRV GDGVQ  RNHET VFQGMLRKLD
bad            LGPSLTEDQP GPYLAPGLLG SNIHQQGRAA TNSHHG  GAGA METRS  RHSSY PAGTEEDEGM

                                                           BH 1
                130            140       150       160          170        180
bcl-2          LTPFTARGRF AT  VVEELFRD GVNWGRIVAF FEF  GGVMCVE SVNREMSPLV DNIALWMTEY
bclx long      ITPGTAYQSF EQ  VVNELFRD GVNWGRIVAF FSF  GGALCVE SVDKEMQVLV SRIASWMATY
bclx short     ITPGTAYQSF ED  TFVDLY
bax            T-DS-PREVF FR  VAADMFSD GNNWGRVVAL FYF  ASKLVLK ALCTKVPELI RTIMGWTLDF
bak            PTAENAYEYF TK  IATSLFES GINWGRVVAL LGF  GYRLALH VYQHGLTGFL GQVTRFVVDF
A1             T----ARIIF NQ  VMEKEFED GINWGRIVTI FAF  GGVLL-K KLPQEQIALD KQVSSFVAEF
mcl-1          IKNEDDVKSL SR  VMIHVFSD GVTWGRIVTL ISF  GAFVAKH LKTINQESCI EPLAESITDV
bad            EEELSPFRGR SR  SAPPNLWA AQRYGRELRR MSD  EFEGSFK GLPRPKSAGT ATQ

                                           BH2
                   190      200             210        220        230
bcl-2          LNR  HLHTWIQ DNGGWDAFV E  LYGPSMRPLF DFSWLSLKTL LSLALVGACI TLGAYLSHK
bclx long      LND  HLEPWIQ ENGGWDTFV D  LYGNAAESRK GQERFNRWFL TGMTVAGVV- LLGSLFSRK>
bax            LRE  RLLGWIQ DQGGWDGLL S  YFGTPTW>
bak            MLH  HCARWIA QRGGWVAAL N  LGGPILNVLV VLGV-VL>
A1             IMN  NTGEWIR QNGGWEGFI K  KFEPK-SGWL TF>
mcl-1          LVR  TKRDWLV KQRGWDGFV E  FFH-V-EDLE -GGI-RN-VL LAFAGVAGV- GAGLYLIR>
bad            MRQ  SAGWTRI IQSWWDRN     LGKGGSTPSQ >
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Figure 1. Sequence alignment of bcl-2 family proteins. Conserved amino acids are indicated in bold face type. The boxes indicate conserved BH1, BH2, BH3 domains and domain A.

active promoter, is GC-rich and TATA-less with multiple start sites and seven consensus binding sides for the Sp1 transcription factor. The P2 promoter is 1.3 kb downstream of P1 and includes a CCAAT box, a TATA element and two discrete initiation sites. Additionally, the P2 promoter contains an octamer motif which is similar to the one found in immunoglobulin promoters and identical to the octamer in the simian virus 40 (SV40) gene. A 1.3-kb negative regulatory element (NRE) in the 5′ untranslated region of *bcl-2* lies between the P1 promoter and the translation start site and includes the P2 promoter. This NRE appears to be important for the stage-specific expression of bcl-2 in lymphoid cells [36].

The human bcl-2 consists of 239 amino acids and includes a 19-amino acid hydrophobic carboxy-terminal end which functions as a membrane anchor [37]. The transmembrane domain may serve to optimize bcl-2 function [38, 39], but it has been shown in deletion mutants that cell death suppression by bcl-2 can be maintained in the absence of the membrane-spanning domain [40, 41]. Immunoelectron microscopic studies have demonstrated that the bcl-2 protein preferentially localizes within the mitochondria at points where the inner and outer mitochondrial membranes are closely opposed, the endoplasmic reticulum and the nuclear envelope [19, 42–48].

Members of the bcl-2 family of proteins share two conserved regions which are referred to as BH1 and BH2 [30, 31, 49] or domains B and C [50, 51], respectively. The BH1 domain extends from amino acid residue 136 to 155 and BH2 from residue 187 to 202. Both BH1 and BH2 domains appear to be important for mediating the physical interaction between bcl-2 and other family members, most notably the dominantly acting death accelerator, bax [31, 34, 50, 52, 53]. The BH1 and BH2 domains do not appear to mediate bcl-2 homodimerization. Rather, formation of bcl-2 homodimers appears to be mediated by a conserved NH2 terminal region spanning residues 11–33 and referred to as domain A [50]. It has been speculated that the ability of bcl-2 to suppress cell death is a function of the relative proportion of bcl-2 homodimers and bcl-2/bax heterodimers.

Mice transgenic for *bcl-2* developed B-cell hyperplasia [54, 55] initially followed by malignant lymphoma developed after a latency period of 15 months [56]. These lymphomas were clonal B cell malignancies, and half of the tumours demonstrated clonal rearrangements of c-*myc* [56, 57]. These findings support a role for cell death

suppression during multistep lymphomagenesis [5, 58, 59].

Complete absence of bcl-2 protein in *bcl-2*−/− mice does not interfere with normal embryonic development, but several abnormalities become evident postnatally [18, 60, 61]. The mice display growth retardation, small ears and polycystic kidneys. The thymus and spleen become atrophic in the bcl-2-deficient mice secondary to massive lymphocyte apoptosis. The majority of mice die within several months, with kidney failure representing the most likely cause of death.

Although bcl-2 is subject to both tissue-specific and developmental regulation, little is known about the molecular mechanisms of its function. Recent evidence suggests that bcl-2 may modulate intracellular signalling events associated with cell death induction. In this regard, a sustained modest increase in intracellular Ca^{2+} is frequently implicated as an important cell death signalling mechanism [62]. This Ca^{2+} signalling results initially from depletion of Ca^{2+} from the endoplasmic reticulum followed by the capacitative influx of Ca^{2+} across the cytoplasmic membrane. Bcl-2 has been shown to inhibit depletion of Ca^{2+} from the endoplasmic reticulum and thereby prevent capacitative Ca^{2+} entry into the cell [21, 22]. Additionally, bcl-2 also appears to actively inhibit the intranuclear accumulation of Ca^{2+} following cell death induction [22].

Reactive oxygen species have also been implicated in the mediation of apoptotic cell death in some experimental systems. In this regard, bcl-2 has been shown to inhibit the generation of reactive oxygen species or to significantly impede downstream oxidative damage resulting from the radicals once they have been generated [42, 52, 64]. However, it has also been demonstrated that bcl-2 can inhibit cell death induction under conditions which preclude the formation of reactive oxygen species [65, 66]. Therefore, the ability of bcl-2 to protect against the damaging effects of free radicals does not appear to account for bcl-2 cell death suppression completely.

The profound conservation of cell death regulatory mechanisms has recently been illustrated by consideration of developmental cell deaths in the nematode worm *Caenorhabditis elegans*. In the nematode, 131 of the 1090 somatic cells undergo programmed death during hermaphrodite development, and the regulation of this process appears to involve only three genes, *ced-3*, *ced-4* and *ced-9* [67]. Gain-of-function mutations involving *ced-9* prevent normal developmental cell deaths, while *ced-9* loss of function is lethal [17, 68, 69]. Sequence comparisons have shown that ced-9 shares 23% identity with the human bcl-2 protein and appears to be, in fact, the nematode homologue of bcl-2. That the human *bcl-2* gene was shown to prevent many of the developmental cell deaths in the nematode illustrates the remarkable evolutionary conservation of cell death regulation in general and bcl-2 function in particular [13, 17].

The nematode cell-death gene *ced-3*, which encodes a cystine protease, has been shown to be required for almost all PCD in *C. elegans* [67]. The mammalian interleukin-1β-converting enzyme (ICE) was identified by homology search and exhibits functional similarities in the molecular mechanism of apoptosis [70, 71]. A similar protein is encoded by the developmentally regulated mouse gene Nedd2 [63]. Recently, another ICE-like protease, CPP32/YAMA [72–75] has been cloned, which cleaves the poly[adenosine diphosphate(ADP) ribose] polymerase DNA repair enzyme following apoptosis induction. These proteases are suggested to lie near the apex of the apoptosis pathway and their ability to mediate apoptosis can be blocked by bcl-2 or members of the bcl-2 family [76]. The molecular basis of this interaction has not been characterized.

Specific viral proteins, including the Epstein-Barr virus BHRF1 [77] and adenovirus 19K E1B proteins [78], also show significant sequence similarities with bcl-2. Expression of these viral proteins has been shown to confer resistance to cell death induction in experimental systems. Again, these findings imply impressive conservation of cell death regulation by bcl-2-related proteins.

Together, these studies may indicate that bcl-2 may suppress cell death by multiple independent mechanisms. However, it is also plausible, and arguably more satisfying, that from a more complete understanding of bcl-2 a 'unified theory' of bcl-2 function will emerge.

Bax

The bcl-2-associated X protein, bax, was first identified as a 21-kD protein that coimmunoprecipitates with bcl-2 [30]. *Bax* is a six exon, 4.5-kb gene and maps to 19q13.3–q13.4 [79]. *Bax* shares 21% identity and 43% similarity with *bcl-2*. The most conserved regions between the two molecules are the BH1 and BH2 domains encoded by exons 4 and 5, respectively. The junction of bax exons 5 and 6 is identical to the exon 2 and 3 junction in the *bcl-2* gene [30].

A complex pattern of alternative RNA splicing predicts three different splice variants of bax. *Bax-α* has a 1.0-kb RNA that encodes a 192-amino acid, 21-kD protein with a transmembrane hydrophobic region. *Bax-β* has a 1.5-kb RNA that encodes a 218-amino acid, 24-kD protein that lacks the transmembrane segment and is presumably a cytosolic form of bax. *Bax-γ* lacks the 53 bp exon 2, and consequently the reading frame in exon 3 is shifted. Alternative splicing of intron 5 in *bax-γ* produces a 1.0- and 1.5-kb transcript [12]. More recently, a *bax-δ* cDNA clone was isolated which results from splicing of exon 2 to exon 4 and retains the carboxy-terminal transmembrane anchor as well as the

BH1 and BH2 domains [79]. The functional significance of these individual bax variants is, in large part, unknown.

Initial mutagenic analysis of the BH1 and BH2 domains of bcl-2 and bcl-x_L revealed that mutations which disrupt bcl-2/bax or bcl-x_L/bax heterodimer formation also resulted in loss of bcl-x_L and bcl-2's ability to inhibit apoptosis [31, 49]. However, recent data suggest that not all bcl-2 mutants that retained the ability to heterodimerize with bax in vitro can neutralize bax mediated cytotoxicity [50] and that interaction between bax and bcl-x is not required for bcl-x to exert its death-repressing activity [80]. Bax can also heterodimerize with other bcl-2-related proteins, including MCL-1 and A1, as shown by the yeast two hybrid assay [53]. These data suggest that bcl-2 family members function in part through protein-protein interactions and that susceptibility to apoptosis is determined by multiple competing dimerizations in which bax is a common partner.

Enforced bax expression may overcome bcl-2-mediated cell death suppression and result in accelerated apoptotic cell death following a death inducing stimulus [30]. It has been suggested that the relative amounts of bcl-2 and bax may be considered as a 'rheostat' which determines the susceptibility of a cell to undergo apoptosis [52]. According to this scheme, when bcl-2 is in excess, bcl-2 homodimers predominate and protect the cell from apoptosis. Conversely, when bax is in excess, bax homodimers predominate, and the cell becomes susceptible to apoptosis following exposure to an apoptotic signal.

Immunohistochemical analysis of bax protein tissue distribution showed that bax is more ubiquitously expressed compared with bcl-2 [81]. Although there is some overlap between bax and bcl-2 proteins their subcellular distribution is nonoverlapping within several tissues. For example, bax immunostaining was located in the base of the crypts of the small intestinal mucosa and surface epithelial cells in the colon. In contrast, bcl-2 protein staining was mainly detected in the absorptive epithelium of the small intestine and the base of the colonic crypts. In the prostate strong bax immunostaining was detected in the androgen-dependent secretory epithelium, whereas bcl-2 was only expressed in the androgen-independent basal cells. Differential expression was also seen in the lymph nodes, where bax is expressed in the germinal center lymphocytes known to exhibit high levels of apoptosis, while bcl-2 expression was expressed primarily by the interfollicular lymphocytes [81].

The regulation of cell death displays lineage-specific aberrations in *bax* knockout mice [82]. The thymuses and spleens of *bax*-deficient mice were hyperplastic secondary to an accumulation of T and B lymphocytes. However, the ovaries contained unusual atretic follicles with excess granulosa cells. Additionally, the male *bax* knockout mice were infertile secondary to disorders of the seminiferous tubules, accumulation of atypical premeiotic germ cells, and absence of mature haploid spermatocytes. These alterations were accompanied by multinucleated giant cells and dysplastic changes. Therefore, developmental absence of bax protein can result in either hyperplasia or hypoplasia in a cell type-dependent manner.

The expression of *bax* can be modulated at the transcriptional level by p53 [83]. The M1 myeloid leukemia cell line transfected with a temperature-sensitive p53 mutant expression vector exhibits a rapid increase in *bax* mRNA within 4 h of shifting cells to the permissive temperature [84]. Additionally, the steady-state level of bax protein is lower in cells obtained from p53 knockout mice [85]. *Bax* mRNA was also upregulated following apoptosis induction by ionizing radiation but only in cell lines that possess wild-type p53 [84]. The *bax* gene promoter was shown to contain four p53-binding sites [83]. These sites could be specifically transcriptionally transactivated by p53 in reporter plasmids driven by the *bax* promoter. Together, these data suggest that *bax* may function as a primary response gene in a p53-regulated apoptotic pathway [83]. *Bax* mRNA levels have also been shown to be downregulated in myeloid leukemia cell lines treated with IL-6 and/or dexamethasone [27]. Pulse chase analysis indicated that the half-life ($t_{1/2}$) of *bax* mRNA increased to 24 h in *bcl-2*-transfected 697 pre-B-cell leukemia cells relative to a $t_{1/2}$ of 4 h in control transfected cells [86]. Post-transcriptional regulation of *bax* mRNA steady-state levels by bcl-2 protein was found to be tissue specific [86].

Recent evidence suggests that bax may play a role as a tumour suppressor. For example, bax-α is expressed at high levels in normal breast tissue and is undetectable or expressed at low levels in breast cancers [87]. Furthermore, reduced bax expression was associated with poor response to combination chemotherapy and shorter survival in women with metastatic breast cancer [88]. Direct experimental findings concerning the potential tumour suppressor activity of *bax* have, however, not yet been reported.

Bcl-x

Bcl-x was first identified using a murine *bcl-2* cDNA probe under low stringency conditions to identify *bcl-2*-related genes in chicken lymphoid cells [32]. The *bcl-x* open reading frame displays 44% identity with *bcl-2*. Bcl-x appears to be capable of mediating the same spectrum of interactions with members of the bcl-2 family as that observed with bcl-2 when analysed by the yeast two-hybrid system [89]. Two distinct human *bcl-x* cDNAs have been identified [32]. *Bcl-x_L* (long form)

contains an open reading frame encoding a 233-amino acid, 21-kD protein and includes the BH1 and BH2 domains. The *bcl-x_L* cDNA was found to be colinear with the genomic sequence, denoting the absence of mRNA splicing. *Bcl-x_S* (short form) encodes a 170-amino acid, 19-kD protein. The carboxy-terminal 63 amino acids encoding the BH1 and BH2 domains are deleted from a 5′ splice site within exon 1 of the *bcl-x* gene [32]. A third alternative splice variant of *bcl-x* has been isolated from a murine cDNA library, *bcl-x_β* [90]. *Bcl-x_β* encodes a 209-amino acid protein that results from an unspliced first coding exon and lacks the carboxy-terminal 19 hydrophobic amino acids necessary for transmembrane insertion.

Bcl-x levels are generally higher than bcl-2 levels in all tissues examined except for lymph nodes where bcl-2 is predominant [91]. High levels of bcl-x_L are found in cells of the central nervous system, kidney and bone marrow [90, 92]. Both bcl-x_L and bcl-x_S, but not bcl-2, are expressed in $CD34^+$, $CD38^-$, lin^- primitive hematopoietic precursors [93]. The subcellular distribution of bcl-x protein is similar to bcl-2 and localizes to mitochondria and nuclear envelope [90].

Bcl-x-deficient mice have been generated using the techniques of homologous recombination [94]. Heterozygous mice develop normally, while homozygous knockout mutants die at approximately day 13 of gestation. The *bcl-x* knockout embryos display extensive apoptosis involving postmitotic neurons of the developing brain, spinal cord, dorsal root ganglia and hematopoietic cells in the liver. Additionally, lymphocytes from *bcl-x*-deficient mice showed diminished maturation. The life span of immature lymphocytes, but not mature lymphocytes, was shortened. These observations emphasize the importance of bcl-x in maintaining the viability of cells necessary for the appropriate development of the nervous and hematopoietic systems.

Interestingly, bcl-x_L was shown to confer resistance to apoptosis induction following growth factor deprivation; however, bcl-x_S counteracted the ability of bcl-2 to block apoptosis [32]. Although bcl-x_L and bcl-2 are general inhibitors of apoptotic cell death, several observations suggest that the biological functions of these two proteins are not completely overlapping. The tissue distribution of bax and bcl-x are not identical, and the phenotypes of the corresponding knockout strains of mice are substantially different. Furthermore, it has been shown that WEHI-231 cells can be protected from apoptosis induced by surface IgM cross-linking by enforced bcl-x expression, while enforced bcl-2 expression exerts no such protective effect [95, 96].

Bak

Bak (bcl-2-homologous antagonist/killer) was first cloned from human heart and Epstein Barr-transformed human B cell cDNA libraries [97–99]. There are three closely related *bak* genes (*bak-1*, *2* and *3*) which are located on chromosome 6 (*bak-1*), chromosome 20 (*bak-2*) and chromosome 11 (*bak-3*). The *bak* genes contain at least three exons and span 6 kb. Bak is a 211-amino-acid, 23-kD protein which shares 53% amino-acid identity with bcl-2. It possesses the same hydrophobic carboxy-terminal domain as bcl-2 and bcl-x_L, which suggests that bak is an integral membrane protein. In contrast to bcl-2, bak is expressed at high levels in the kidney, pancreas, liver and fetal heart, as well as in adult brain [99].

Bak was shown to accelerate cell death following IL-3 withdrawal [97, 99], but inhibits apoptosis induced by serum withdrawal and menadione treatment [97].

Bad

Bad is a novel member of the bcl-2 family that was identified as a bcl-2-interacting protein using the yeast two-hybrid system [34]. The full-length *bad* cDNA sequence encodes a novel 204-amino acid protein with a predicted molecular weight of 22 kD. Bad shares only limited homology with known bcl-2 family members in the BH1 and BH2 domains. However, the functionally significant W/YGR triplet in BH1, the W at position 183, the WD/E at the exon junction in BH2 and the spacing between BH1 and BH2 domains is conserved. Unlike many other bcl-2 family members, bad does not contain a transmembrane anchor domain.

Bad was shown to heterodimerize with bcl-2 and bcl-x_L in vivo using coimmunoprecipitation. Bad interaction with either bcl-2 or bcl-x can displace bax from the heterodimers. Significantly, this was shown to reverse the death-repressor activity of bcl-x but not of bcl-2. However, bad does not appear to interact with bax, bcl-x_S, MCL-1 or A1 nor, apparently, does bad form homodimers.

MCL-1 and A1

MCL-1 was identified in the human myeloid leukemia cell line, ML-1, following induction by phorbol 12-myristate 13-acetate (TPA) [33] and A1 was isolated from murine macrophages after GM-CSF-induced differentiation [100]. Several similarities exist between MCL-1 and A1. Both have been classified as early response genes, meaning that there is a rapid onset and decline of expression immediately following differentiation induction [33, 100, 101]. Studies done using yeast two-hybrid assays indicate that both gene products interact strongly and selectively with bax, however, conflicting results have been seen for MCL-1/bcl-2 and MCL-1/bcl-x_L interactions [53, 89]. Both MCL-1 and A1 share homology to bcl-2 in the BH1 and BH2 domains [33, 100], but only MCL-1 has a carboxy-ter-

minal transmembrane anchor domain [101]. In addition, the MCL-1 protein contains PEST (proline glutamatic serine threonine) sequences [33], which are associated with rapid protein turnover and which correlate with its role as an early response gene product [101]. The human *MCL-1* gene maps to chromosome 1 band q21 [102], an area often involved in chromosomal abnormalities in neoplastic and preneoplastic diseases [103–105].

MCL-1 protects against apoptosis induced by constitutive expression of c-*myc* or bax [106]. However, in the 5AHSmyc cell line, MCL-1 overexpression is not as effective as bcl-2 overexpression in preventing myc-mediated cell death [107]. Similar studies to determine the consequences of A1 expression in the context of cell death regulation have not yet been reported. The correlation of GM-CSF and LPS-induced differentiation with A1 upregulation suggests A1 could potentially function as a cell death suppressor [100].

It has been proposed that MCL-1 may function as an alternative to bcl-2 in situations where bcl-2 cannot block apoptosis or in tissues lacking bcl-2 expression. For example, in normal peripheral blood B cells treated with agents which promote survival (IL-4, anti-μ and TPA) or agents which enhance rates of cell death (TGF-β1 and forskolin), upregulation of MCL-1 correlates with cell survival and downregulation of MCL-1 precedes cell death, while levels of bcl-2 remain unchanged under the same conditions [108].

Additionally, the tissue distribution of MCL-1 and bcl-2 expression shows signficant differences such as brain and spinal cord neurons in which bcl-2 predominates compared to skeletal muscle, cardiac muscle, cartilage and liver where MCL-1 predominates [109]. Similarly, MCL-1 levels in normal lymph nodes are highest in germinal centers, where the rate of apoptosis is high. In contrast, bcl-2 is most intense in the mantle zone. It has been postulated that MCL-1 temporarily blocks cell death until death suppressors such as bcl-2 are upregulated [110]. In contrast, A1 expression displays a rather limited tissue distribution and appears to be confined to hemopoietic tissues, including helper T-cells, macrophages and neutrophils [100].

Summary

The realization that the *bcl-2* proto-oncogene functions to suppress cell death [37] and that the deregulated expression of bcl-2 contributes to multistep lymphomagenesis by cell death suppression [54, 56], and not proliferation enhancement, has stimulated widespread interest in the molecular regulation of cell death. This interest has led to an appreciation that bcl-2 is able to render many cell types resistant to cell death induction in a variety of experimental systems. It seems likely that the ability of bcl-2 to suppress cell death will be dependent on its physical interactions with other protein effector molecules. In fact, this rationale formed the basis which resulted in the identification of *bax* and the realization that *bcl-2* is a member of a multigene family of cell death regulators. So what was once considered a passive event, to many not worthy of further scientific inquiry, is now recognized as complexly regulated and relevant to many fundamental biological processes. It is anticipated that with a more detailed understanding of the mechanism(s) of cell death regulation by bcl-2 and its family members, interventions will be conceived and implemented for the benefit of patients suffering from diseases resulting from disordered cell death.

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